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(54) Plasmid for HIV indicator cell lines

(57) A plasmid comprises (1) a replicon for extrachromosomal expression in eukaryotic cells of a linked gene, (2) a gene which codes for a dominant selectable marker and (3) a transactivatable promoter element of an HIV Long Terminal Repeat (LTR) operably linked to the LacZ gene in a manner effective to provide transcription and expression of the LacZ g n following transactivation of the promoter element. When HIV-responsive cells are transfected with such a plasmid they can be used as an indicator of HIV infection (which would result in transactivation of the promoter, leading to expression of the LacZ gene, which is detectable colorimetrically).

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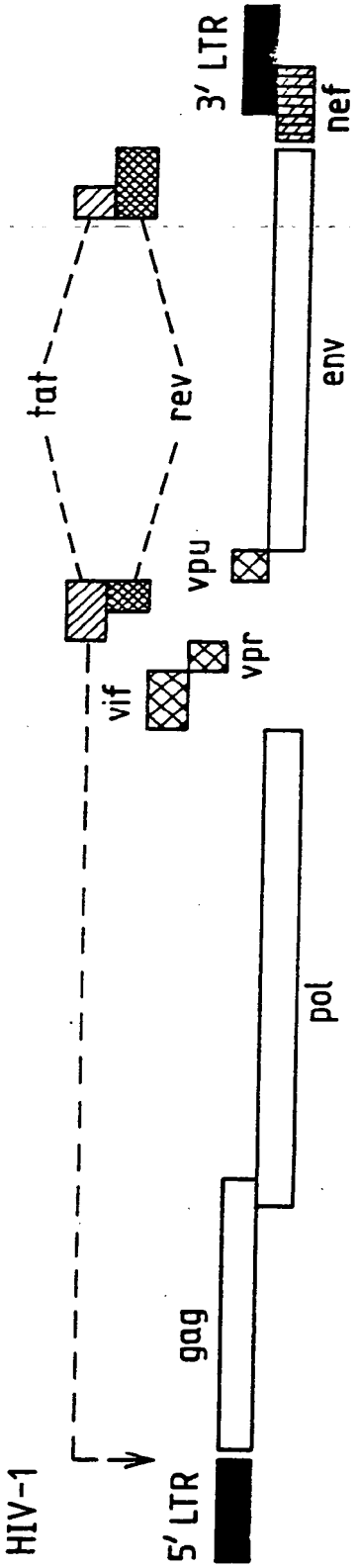
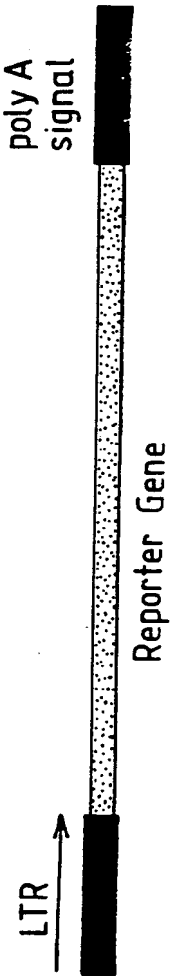


Fig. 1



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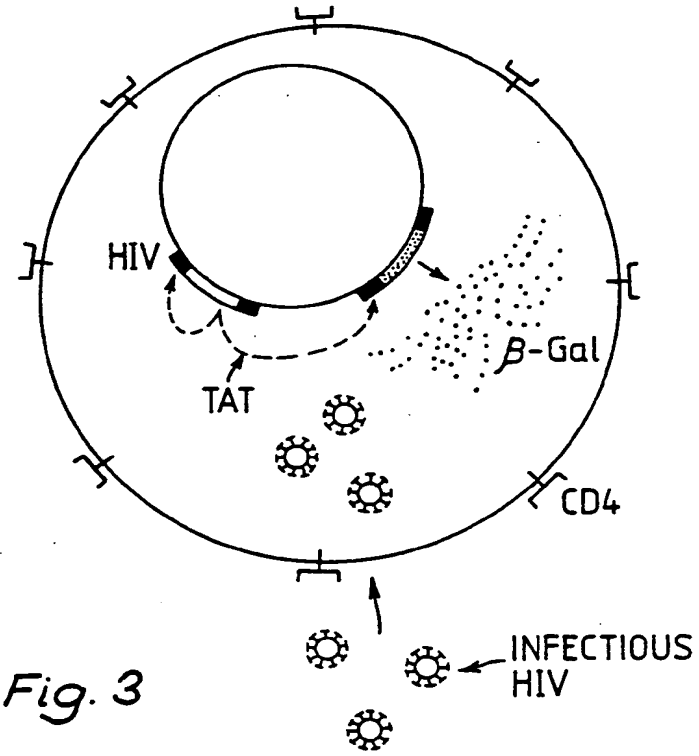

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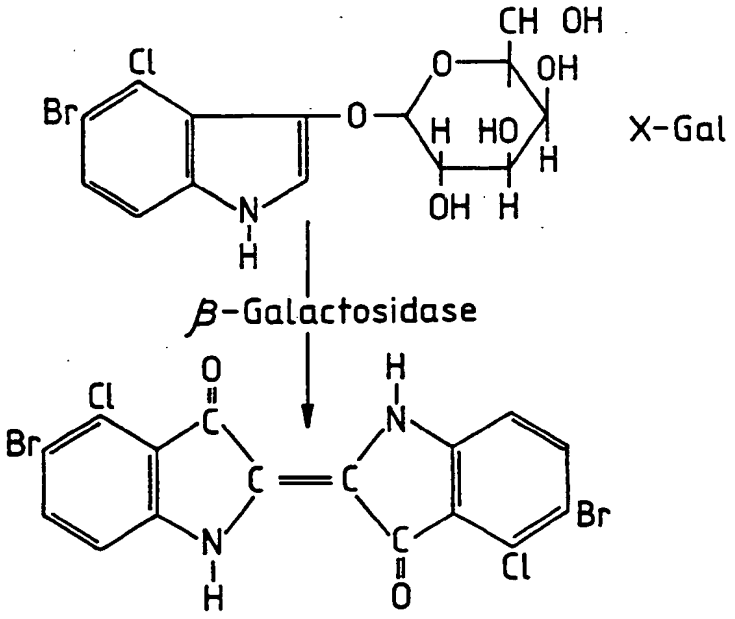

Fig. 4

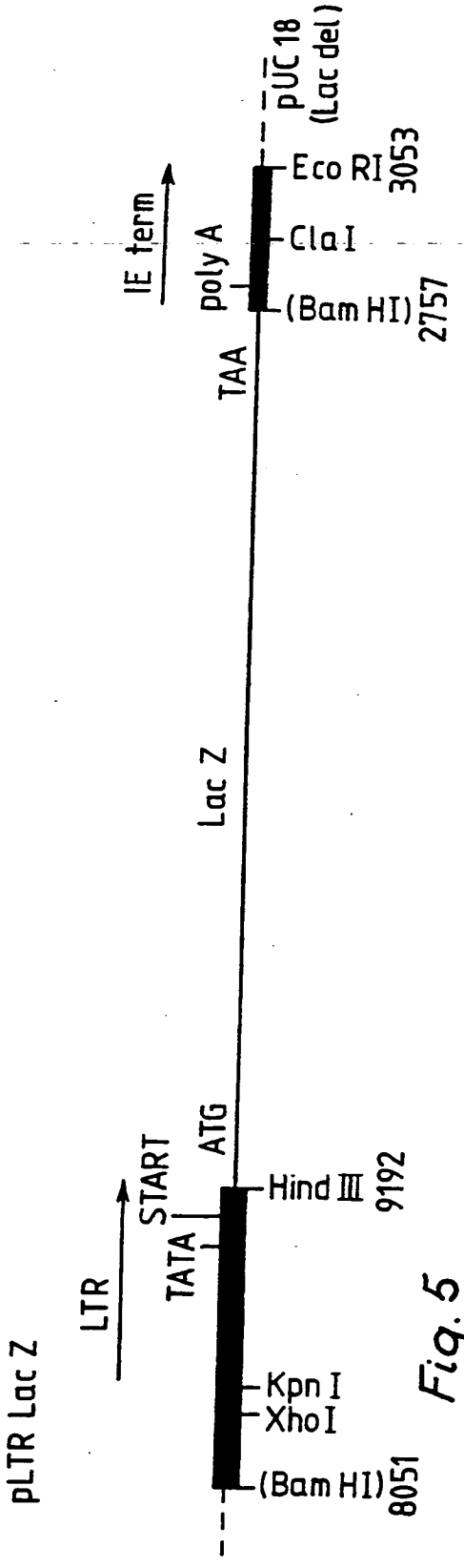

Fig. 5

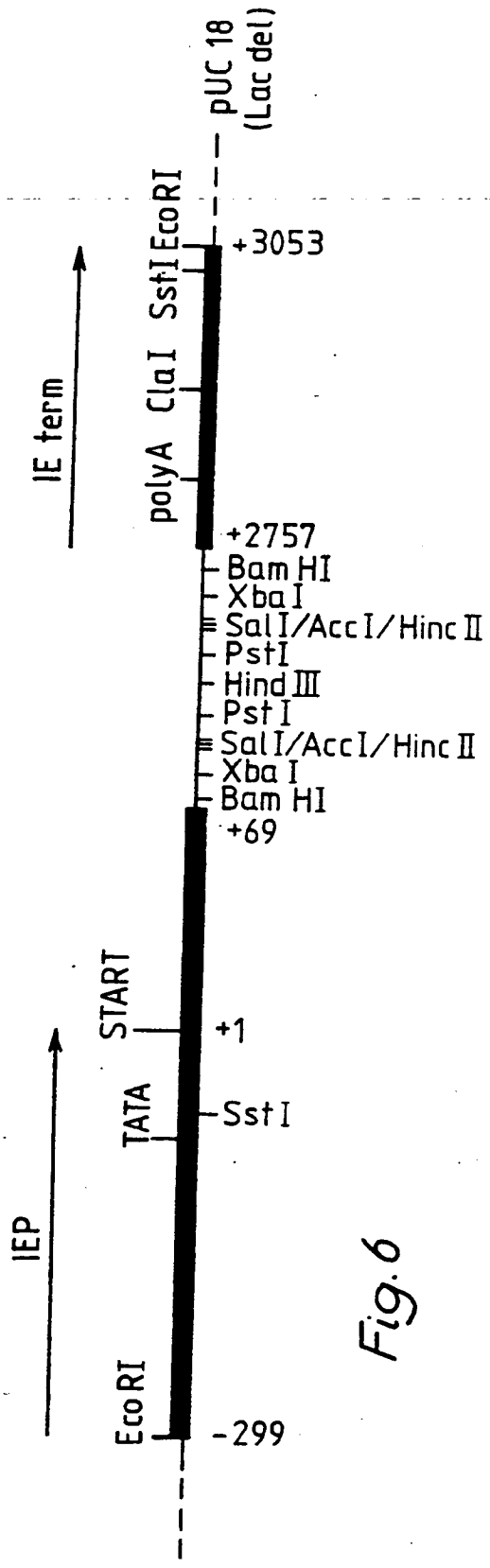

Fig. 6

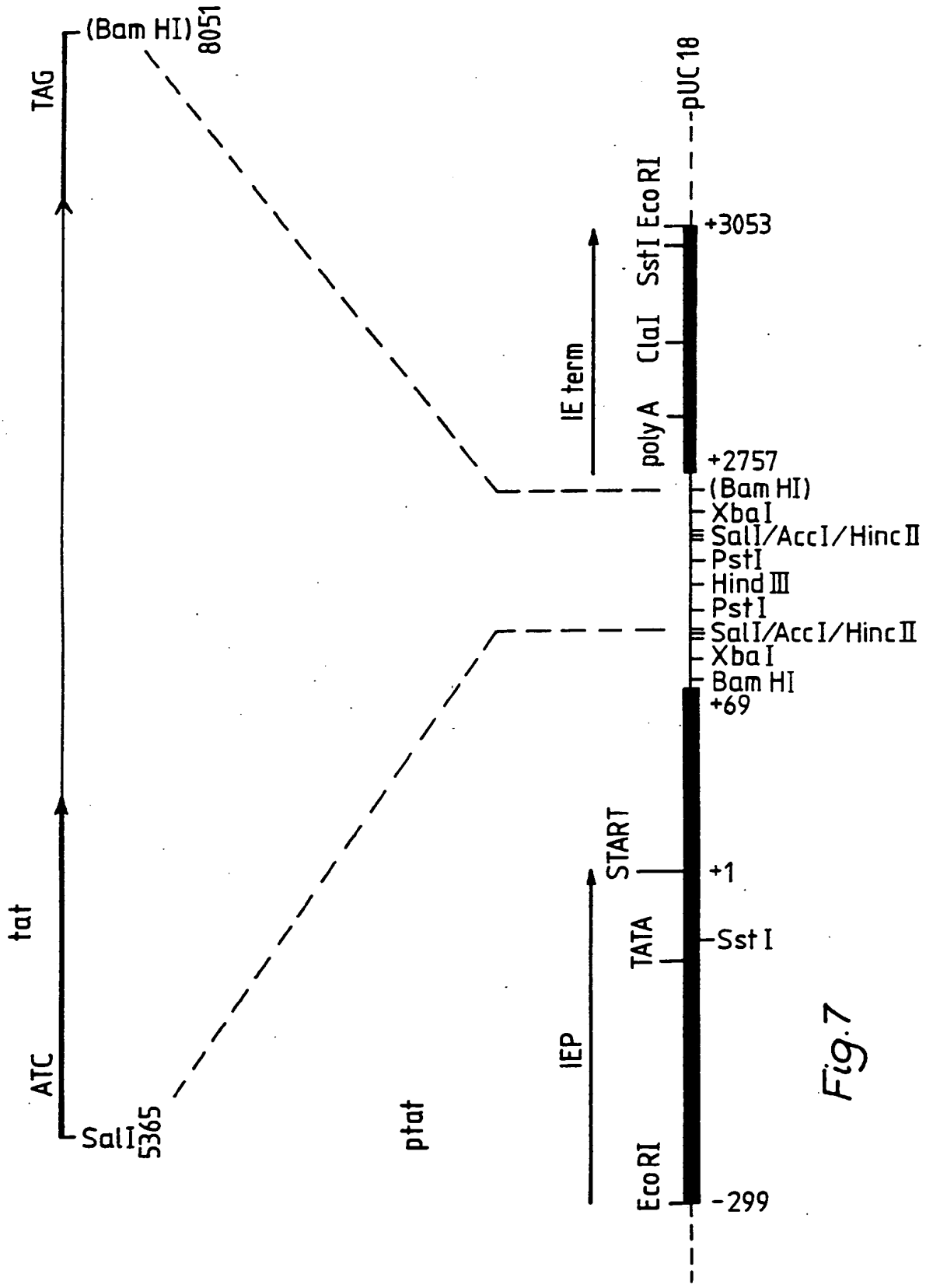

Fig.7

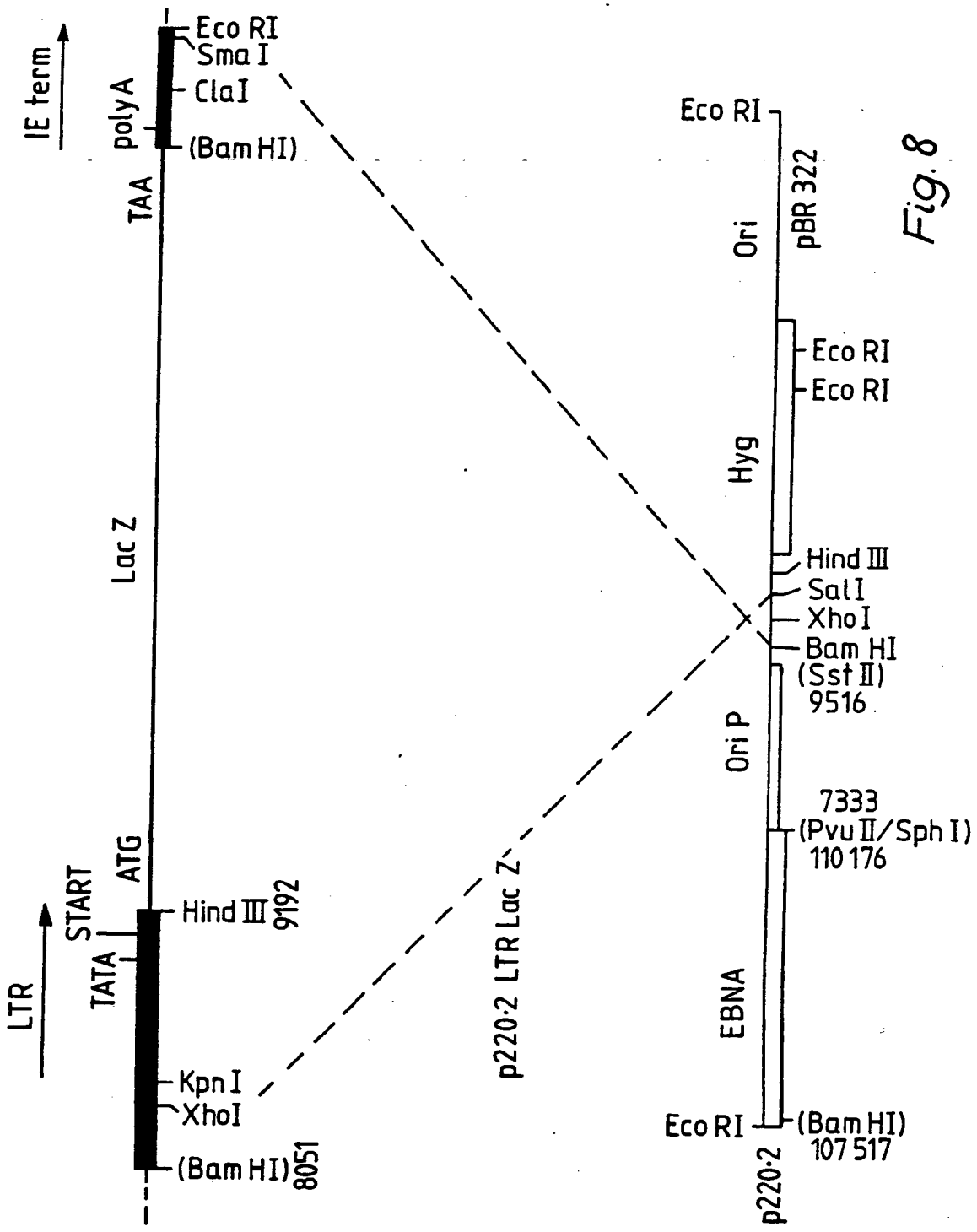

Fig. 8

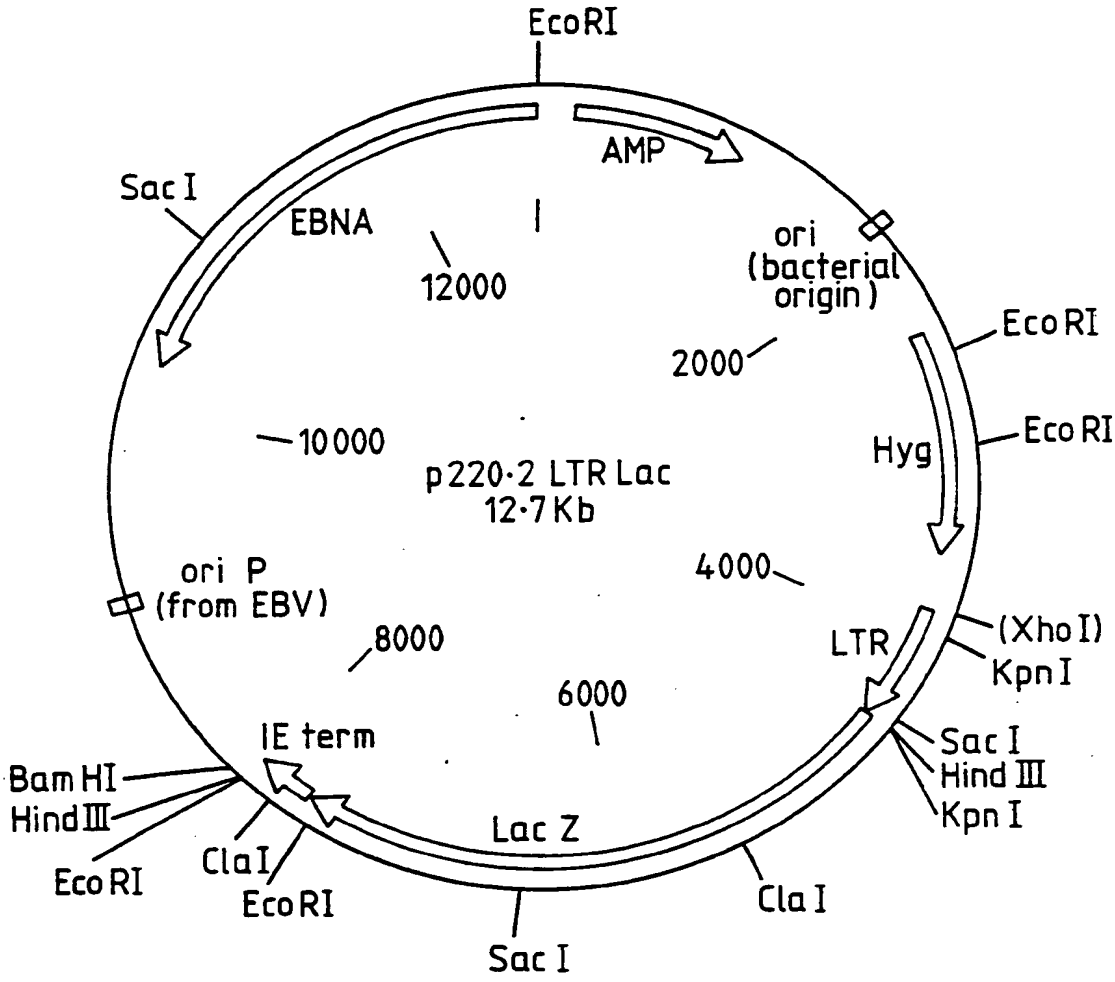

Fig. 9

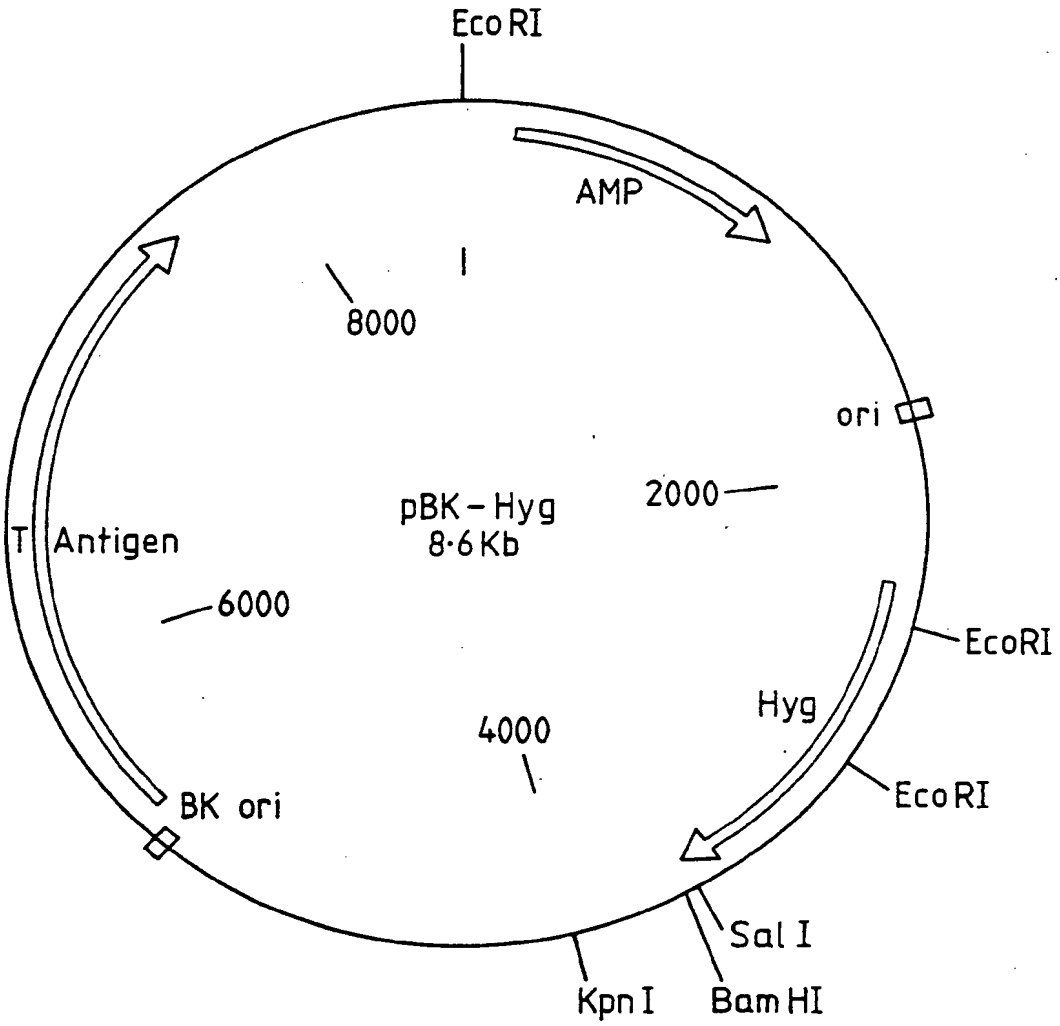

Fig. 10

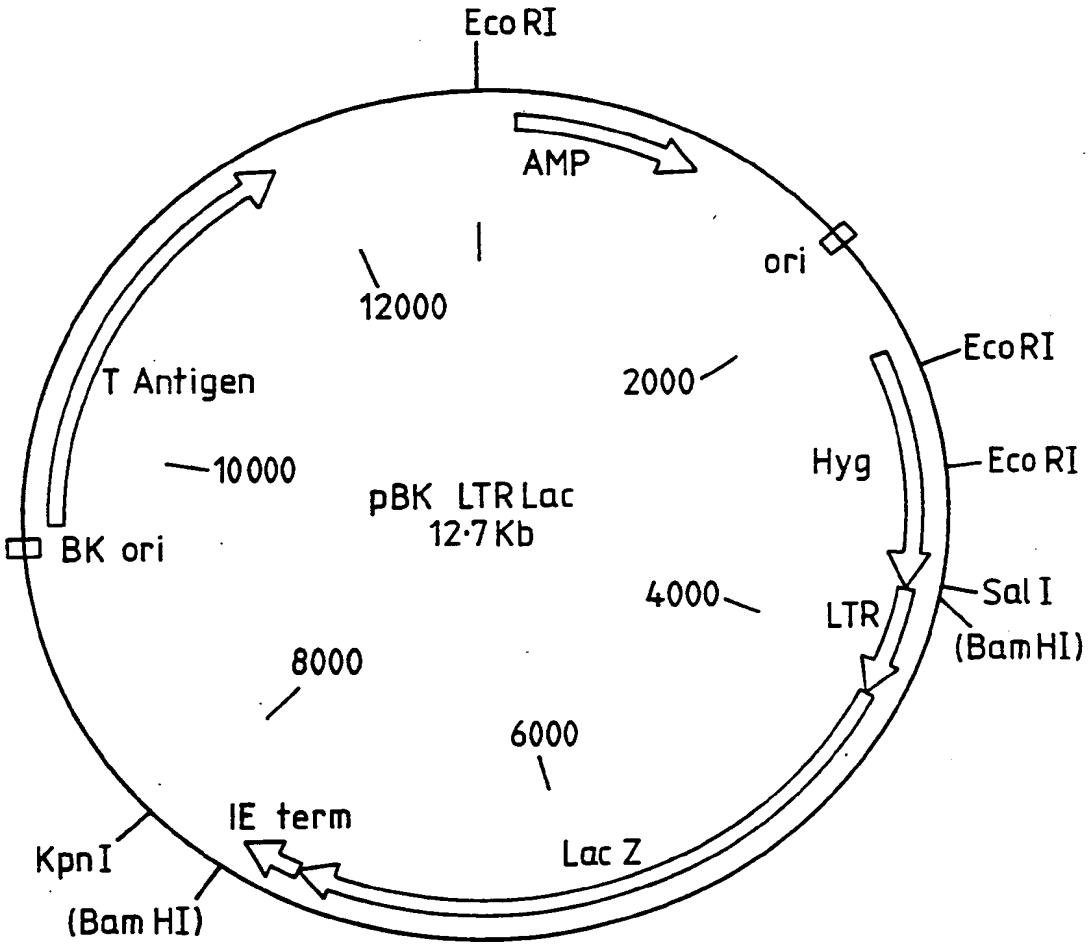

Fig. 11

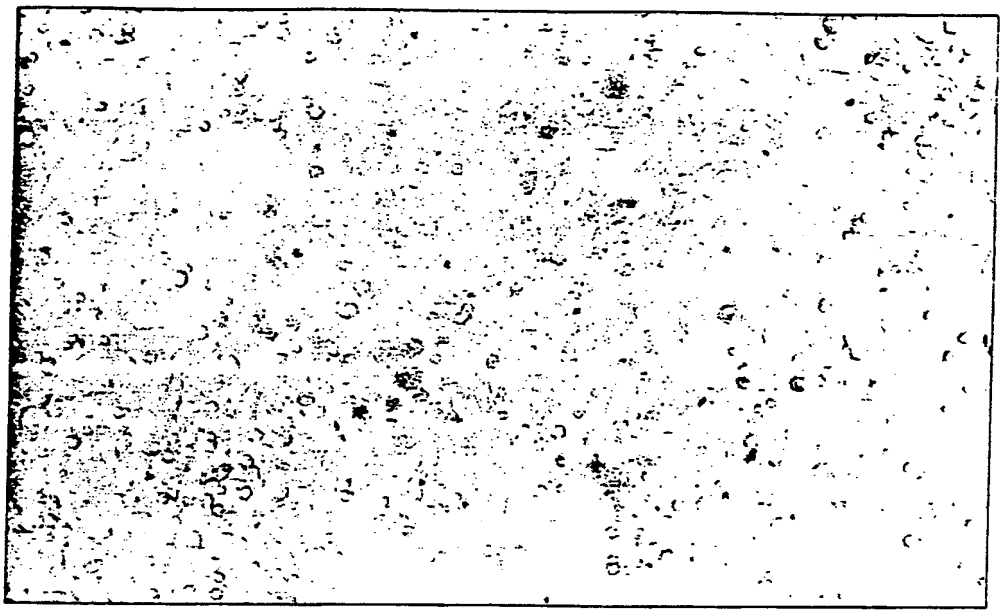

Fig. 12

Fig. 13

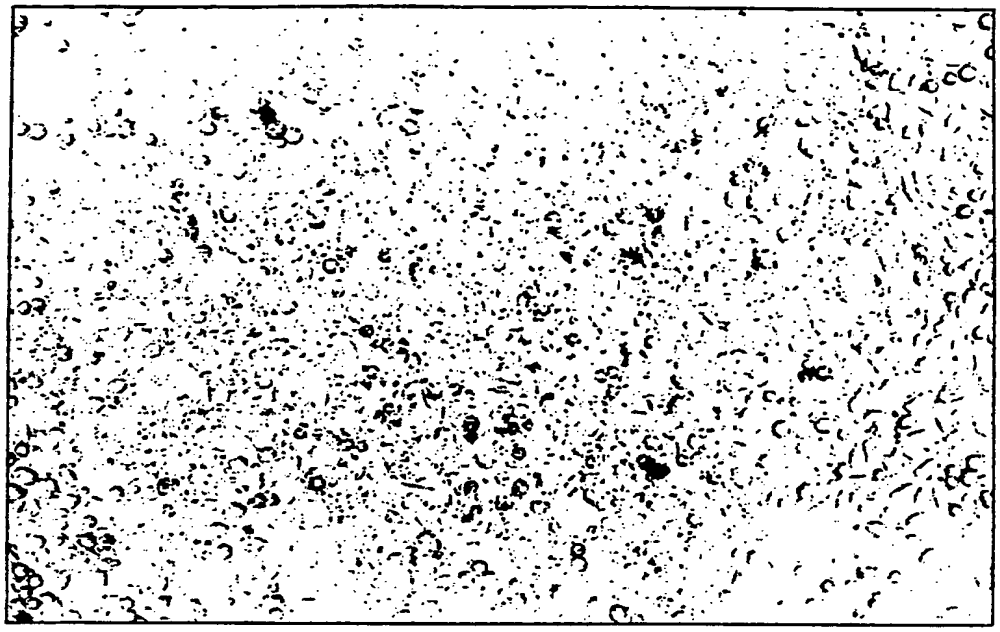

Fig. 14

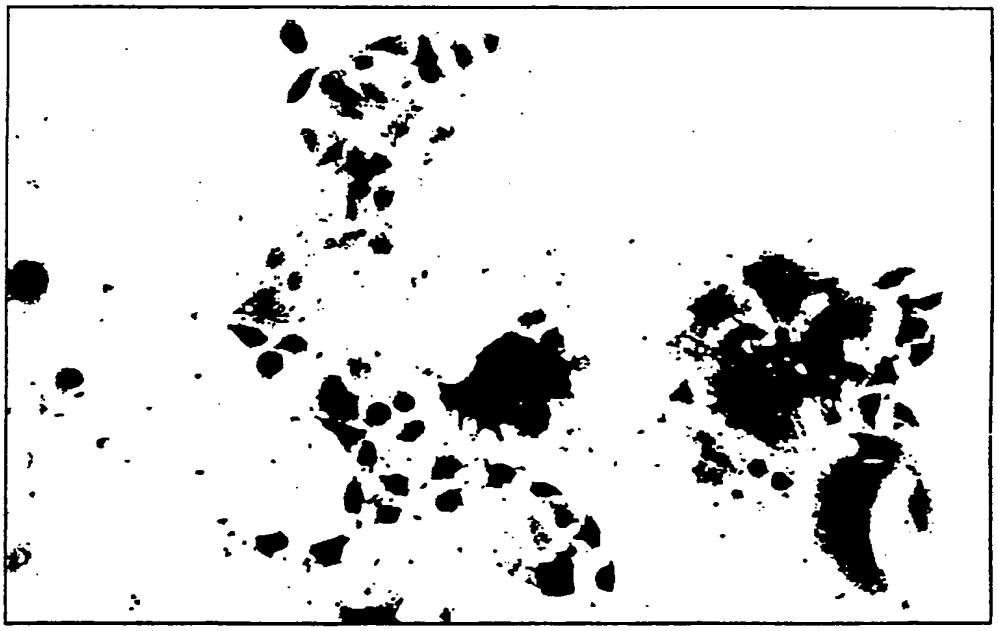

Fig. 15

HIV INDICATOR CELL LINES

This invention relates to HIV indicator cell lines, i.e. cell lines useful for the assay of patient samples for a human immunodeficiency virus, especially HIV-1, and to plasmids useful for transfecting an existing cell line to enable it to become HIV-indicating.

Introduction

Current procedures for visualising HIV infected cells and for quantitative measurement of infectious HIV particles are technically difficult and inconvenient. Immunoassay and dilution to give a 50% infective dose (ID_{50}) have been used frequently (McDougal et al., 1985). Plaque assays have been devised in which HIV-susceptible lymphocytic cell lines are attached to the surface of polylysine-coated dishes and foci of infection identified either by cytopathic effects such as syncytium formation (Harada et al., 1985; Nara and Fishinger, 1988), immunofluorescence (Harada et al., 1985) or immunostaining (Matsui et al., 1987). Following the demonstration that cells non permissive for HIV could be rendered susceptible to HIV infection by the stable incorporation and expression of an exogenous CD4 antigen gene (Madden et al., 1986), adherent HIV infectable HeLa CD4 cell lines have been constructed (Madden et al., 1986; Chesebro and Wehrly, 1988) and used to develop a sensitive quantitative assay for HIV infection (Chesebro and Wehrly, 1988). Foci of infection were detected by immunofluorescence using a monoclonal antibody reactive with HIV envelope protein.

Summary of the invention

We have developed indicator cell lines based on $CD4^+$ HeLa cells which allow much clearer visualisation of foci of HIV infection by means of a simple quick and direct colour assay. The indicator cell lines are stably transfected with a recombinant plasmid vector containing the *E. coli* β-galactosidase gene (LacZ) under the control of the HIV LTR promoter. When these cells are infected by HIV, production of the transactivator

tat induces expression of the LTRLacZ construct, allowing infected cells to be visualised by the blue colour generated following addition of the chromogenic substrate X-Gal. The invention arises from these findings and reasonable extrapolations therefrom.

In an important aspect the invention provides a plasmid comprising (1) a replicon for extrachromosomal expression in eukaryotic cells of a linked gene, (2) a gene which codes for a dominant selectable marker and (3) a transactivatable promoter element of an HIV Long Terminal Repeat operably linked to the LacZ gene in a manner effective to provide transcription and expression of the LacZ gene following transactivation of the promoter element.

The invention further provides HIV-responsive cells, especially cells which are CD4-positive, transfected with a plasmid of the invention as defined above.

In another aspect the invention includes a method of assay of HIV which comprises incubating a sample, suspected of being HIV infected, to cells of the invention under conditions allowing transactivation of the promoter element of the LTRLacZ construct by HIV present in the sample, and assaying the beta-galactosidase produced.

Description of preferred embodiments

In preferred aspects, which can be considered separately or in any feasible combination:

1. The LTRLacZ construct (3) is in the same relative orientation as the replicon (1).

2. The marker gene (2) is in the same relative orientation as the replicon (1).

3. The replicon (1) comprises an origin of eukaryotic cell replication and a nuclear antigen gene.

4. The replicon (1) is from human papovavirus BK (most preferred) or Epstein Barr Virus.

5. The marker gene (2) is a hygromycin or neomycin resistance gene.

6. The LTRLacZ construct comprises a polyadenylated termination sequence, most preferably from cytomegalovirus immediate-early gene.

7. When the elements (1), (2) and (3) are all in the same orientation and the papovavirus BK replicon is used, as is most preferred, the efficacy of individual colonies of transfected cells is tested by infecting them with HIV and selecting those which express LacZ well in the presence of HIV and only minimally in the absence of HIV. That is to say, some low level expression in the absence of HIV appears desirable.

8. The HIV-responsive cells are CD-4 positive, i.e. they express the CD4 surface antigen.

9. The CD4 positive cells are mammalian, e.g. HeLa cells or differentiated cells such as haematopoietic, lymphocyte or mononuclear cells.

10. The HIV-responsive, preferably CD4-positive, cells are adherent to surfaces (whereby they can be grown in a monolayer thereon).

11. The plasmid is bacterially replicable.

12. The plasmid is similar to that of Figure 11.

13. The plasmid is similar to that of Figure 9 but with the EBNA nuclear antigen in the opposite orientation to that shown.

14. The patient's sample is of blood or serum.

15. The beta-galactosidase is assayed colorimetrically, preferably on "Xgal" substrate, giving blue plaques.

The basic principles of the indicator cell lines are illustrated in Figs. 1-3. Fig. 1 depicts schematically the HIV genome showing the location of various genes, labelled with their well-recognised abbreviations. Some of the genes are non-contiguous in the genome, but are spliced together in the mRNA. During HIV infection the tat gene product transactivates the 5' LTR promoter, stimulating expression of the viral genes under its control (Fig. 1). The complex mechanism involves interaction with the transactivator receptor element located at the 5' end of the mRNA (for review see Sharp and Marciniak 1989).

When the viral genes downstream of the LTR promoter are replaced by the reporter gene β-galactosidase (LacZ) together with a polyadenylation signal for termination of transcription (Fig. 2), and the construct stably incorporated into the genome of an adherent $CD4^+$ cell line (Fig. 3), HIV infection, leading to the production of tat by the integrated provirus, causes induction of the LTR promoter driving the β-galactosidase gene. Reaction of β-galactosidase with added X-Gal (5-Bromo-4-Chloro-3 indolyl-β-D galactopyranoside, [Pearson et al., 1963]), (Fig. 4), results in the formation of a dark blue precipitate of indigo (indigotin)-based dye inside infected cells.

Construction and Testing of the Recombinant Plasmids

The plasmid pLTRLacZ (Fig. 5) was constructed by joining the HIV LTR promoter (LAV1 seq. no. 8051 (BamHI) to 9192 (HindIII), Wain-Hobson et al., 1985) a gift from Dr. M. Emerman, Institut Pasteur) to the E. coli LacZ gene (pCH110, Pharmacia Ltd.) and the polyadenylation signal element of the major immediate early gene of human cytomegalovirus (HCMV), (seq. no. 2757-3053; Akrigg et al., 1985). The procedure involved initial cloning of the LacZ gene into the multiple cloning site of an expression plasmid, pIEPCSterm (Fig. 6) comprising the promoter (IEP) and poly A signal sequence of the HCMV major IE gene (Akrigg et al., 1985) and the cloning vector pUC18 (Gibco BRL Ltd.) from which the LacZ sequences (2622-445) had been deleted. The IEP promoter was then replaced by the HIV LTR promoter, by destruction of the 3'-BamHI site (BamHI cleavage, filling in with Klenow DNA polymerase and religation), insertion of the LTR Fragment between the 5' BamHI and HindIII sites followed by removal of the IEP sequences using BamHI partial EcoRI digestion and religation after filling in with Klenow polymerase).

pLTRLacZ was tested for transactivation by the tat gene product following co-transfection with the plasmid ptat (Fig. 7), a plasmid in which the HIV genomic tat sequences are expressed under the control of the HCMV IE promoter, 48 hr. after co-transfection of these plasmids into HeLa cells using the

calcium phosphate co-precipitation method (Graham and Van der Eb, 1973), the growth medium was replaced with PBS containing $MgCl_2$ (1mM) and X-Gal (Sigma Ltd.), (200 µg/ml), and incubated for 4 hours at 37°C (Hall et al., 1983). Co-transfection of ptat along with pLTRLacZ was required to induce significant ß-galactosidase expression and formation of blue colour. pIEPLacZ was a positive control using the HCMV immediate early promoter.

Individual cells expressing the transactivated LTRLacZ construct were more clearly visualised by microscopy when a redox mixture (3mM potassium ferricyanide, 3mM potassium ferrocyanide, 1.3mM $MgCl_2$), (Yarborough et al., 1967, MacGregor et al., 1987) was included with X-Gal (200µg/ml) in PBS (pH7.4). Chemical oxidation increases the rate of precipitation of the blue dye more of which is therefore retained within the cell, instead of diffusing out to colour the supernatant fluid, as occurred without the redox mixture.

The LTRLacZ cassette was then cloned into the eukaryotic expression vector p220.2 (originating from Dr. Bill Sugden's laboratory, University of Wisconsin; unpublished by Sugden but described in Young et al., (1988) which contains the origin of replication (ori P) and nuclear antigen gene (EBNA) of Epstein Barr virus (EBV) as well as the dominant selectable marker gene for Hygromycin B resistance (hyg) (Fig. 8). This vector was chosen for three reasons:-

(i) The most commonly used dominant selectable marker gene (Neo) coding for Geneticin resistance could not be employed because it had already been used to stably insert the CD4 gene into both the $CD4^+$ HeLa cell lines used (HT4-6C, Chesebro and Wehrly (1988), and HeLa-T4, Madden et al., 1986).

(ii) The presence of the EBV replicon allows this vector to replicate in human cells as a multicopy plasmid independent of the host cell's chromosomes (Young et al., 1988). This should increase the gene copy number and therefore expression of LTRLacZ in the presence of tat.

(iii) A gene carried by an independently replicating plasmid

is more likely to be expressed in stably transfected cells than if integration into the host cell's genome is required. Many regions of chromosomal DNA are not normally active for gene expression.

As shown in the linearised DNA insertion diagram of Fig. 8 and the circular plasmid diagram of Fig. 9, the recombinant plasmid p220.2 LTRLac was made as follows. LTRLacZ was removed from pLTRLacZ utilising the XhoI site (8473) at the 5' end of the LTR promoter and the SmaI site (3029) at the 3' end of the poly-A signal sequence. This fragment was cloned into the polylinker of an intermediate plasmid to attach a BamHI linker to the SmaI site and then cloned into p220.2 between the SalI and BamHI sites. Because of initial technical difficulties encountered in selecting Hygromycin-resistant colonies following transfection with the EBV-based vector, the LTRLacZ cassette was also cloned into an alternative episomal expression plasmid pBK-Hyg (Fig. 10), based on the replication machinery (T antigen gene plus origin of replication) of human papovavirus BK (GS strain), (Akrigg et al., 1981). Episomal BK vectors have been shown to replicate efficiently in human cells, independent of the host cell's chromosomes (Milanesi et al., 1984, Grossi et al., 1988). pBK-Hyg was constructed by removing the EBNA and ori P sequences from p220.2 by BamHI/partial EcoRI digestion followed by replacement with the EcoRI-BamHI fragment of pAR16 (Akrigg et al., 1981) equivalent to the BKV sequence 1-4861 comprising the ori and T antigen coding sequences (Yang and Wu, 1979).

To construct pBK-LTRLac (Fig. 11), the LTRLacZ cassette BamHI (8051)-SmaI (3029) was first cloned into an intermediate plasmid to add a BglII linker to the SmaI site, then removed by BglII digestion (utilising the BglII site (8627) at the 5' end of the LTR promoter) and cloned into the BamHI site of pBK-Hyg.

Both recombinant plasmid vectors were tested for transactivation of LTRLac by co-transfection with ptat followed by transient expression in HeLa cells before proceeding to use them to stably transfect $CD4^+$ HeLa cell lines.

Construction of the Indicator Cell Lines

All cells were maintained in Dulbecco's MEM (DMEM) containing 10% foetal calf serum, glutamine 2mM, Penicillin (50 IU/ml) and Streptromycin (50µg/ml). Two different $CD4^+$ HeLa cell lines were used as recipients for LTRLacZ-expressing recombinant plasmids: HT4-6C (Chesebro and Wehrly, 1988) was transfected with p220.2-LTRLac (Fig. 11)1; HeLa T4 (Madden et al., 1986) was transfected with pBK-LTRLac (Fig. 13). Colonies resistant to Hygromycin B (Sigma Ltd.) (50µg/ml and 100µg/ml respectively) were selected after a 48-72 hour expression period. In the case of the HeLa - T4 strain; the selective medium also included Geneticin (Sigma Ltd.), (1000 µg/ml). Selected Hygromycin-resistant colonies were picked, expanded with continued drug selection and tested for expression of LTRLacZ when co-transfected with ptat.

HT4-6C-p220.2 LTRLac clone (22) 3 was found to be efficiently transactivated by tat. As shown by the number of blue cells produced after straining with X-Gal in the absence of ptat, blue cells were very rare. HeLa T4-pBK-LTRLac clones (22)3 and (22)4 were also found to be efficiently transactivated by tat. For these two clones, the number of blue cells observed in the absence of tat was slightly higher than for clone (22) 3. The three cloned cell lines were maintained in DMEM plus supplements containing the same drug concentrations used for selection. Clone (22) 3 was also checked for resistance to Geneticin (1000µg/ml).

HIV Infection of Indicator Cell Lines

The three stable cell lines were initially infected with HIV-1 (strain GB8) by co-cultivation with HIV-infected cells of the lymphoid cell line JM. Approximately 5×10^5 cells of each indicator cell line were inoculated into a $25cm^2$ tissue culture flash containing 10ml of DMEM plus supplements but no Hygromycin or Geneticin. After incubation overnight, 3ml of medium was replaced by 3ml of RPMI medium containing HIV-infected or uninfected JM cells (approximately 10^6/ml). After a further 24

hour incubation, the JM cells were removed and replaced with fresh DMEM, followed by incubation for 4-6 days. Growth medium was then removed and the remaining adherent cells washed with 10ml of PBS. X-Gal (200µg/ml) in 10ml of PBS containing potassium ferricyanide (3mM), potassium ferrocyanide (3mM) and $MgCl_2$ (1.3mM) was added and flasks incubated for 2-4 hours at 37°C and then overnight at room temperature. Cells were fixed for photography by removal of the X-Gal solution and addition of 5ml of 2.5% glutaraldehyde in PBS.

Fig. 12 shows HT4-6C-p220.2 LTRLac clone 3 cells incubated with uninfected JM cells (control); Fig. 13 shows the same cell line incubated with HIV-infected JM cells. In flasks containing HIV many dark blue foci of infection were visible after 2 hours incubation with X-Gal. The stained cells were arranged either as closely associated groups or as complexes of fused cells similar to the syncitia formed during HIV infection of lymphoid cells. Fig. 14 shows HeLa-T4-pBK-LTRLac clone (22) 4 incubated with uninfected JM cells (control). Fig. 15 shows the same cell line incubated with HIV-infected JM cells. Clone (22) 3 gave very similar results. All three clones appeared to be infected and to produce blue cells with equal efficiency. In the case of clone 3 there was a greater tendency of infected cells to form syncytia.

HT4-6C-p220.2 LTRLac clone (22) 3 was also tested for susceptibility to infection by cell-free virus using supernatants from cultures of HIV-infected JM cells following centrifugation at 2,000g for 15 min. 5ml of HIV-containing medium was added to the monolayer of HeLa indicator cells and virus absorption promoted by shaking each flask slowly on a rotary platform at 37°C for 90 min. The medium was then replaced by 10ml of fresh DMEM and incubation continued for 4-6 days. Staining with X-Gal produced large numbers of dark blue foci of infected cells. Reducing the concentration of cell-free virus by 10 fold reduced the number of foci by considerably more than 10 fold and a similar effect was noted when the concentration of HIV-infected JM cells was reduced.

This tends to suggest that a co-operative effect can operate during infection by high concentrations of HIV particles.

ONPG Assay on HIV Infected Indicator Cell Lines

For quantitation of β-galactosidase activity induced by HIV infection of the three indicator cell lines, o-nitrophenyl-β-galactoside (ONPG) assays were performed (Miller, 1972). After 5 days incubation with HIV infected or uninfected JM cells, the adherent HeLa indicator cells were washed with 10ml of PBS and ONPG (800µg/ml) added in 3ml of Z buffer (60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgSO_4$ pH7.0). After incubation at 37°C for 90 min. glutaraldehyde (final concentration 2.5%) was added, followed after 5 min. by 1.1ml of 1M Na_2CO_3. Samples (1.5ml) of each supernatant were taken, centrifuged to remove particulate matter and their OD_{420} measured against a reagant blank.

The results (Table) demonstrated the considerable stimulation of β-galactosidase by HIV infection, the yellow colours produced with ONPG being clearly visible. The BK vector based clones gave higher background (uninfected) values than the EBV based clone. The relative increase in β-galactosidase activity following HIV infection would have been much greater than the OD_{420} values indicate, when adjusted to allow for the considerable loss of indicator cells from HIV infected cultures due to virus induced cell death. In future experiments OD values will be corrected for differences in cell number by measurement of protein concentration in each flask assayed.

Table: ONPG assay of HIV infected indicator cell lines

	OD420
Uninfected HeLa parent strain (control)	0.034
HT4-6C p220.2 LTRLac clone 3 + JM cells	0.098
HT4-6C p220.2 LTRLac clone 3 + HIV infected JM cells	1.4
HeLa - T4 pBK LTRLac clone (22) 3 + JM cells	0.170
HeLa - T4 pBK LTRLac clone (22) 3 + HIV infected JM cells	1.3
HeLa - T4 pBK LTRLac clone (22) 4 + JM cells	0.221
HeLa - T4 pBK LTRLac clone (22) 4 + HIV infected JM cells	1.4

Applications of the HIV-1 Indicator Cell Lines

HIV infection of the $CD4^+$ HeLa cell lines containing LTRLacZ gives a signal that is clearly visible to the naked eye as the appearance of dark blue foci of infection 2-4 hours after addition of X-Gal. Individual infected cells and large syncitial structures produced by HIV induced cell fusion are easily distinguished by microscopy, the blue stain enhancing the visibility of cellular substructures. Groups of cells comprising foci of infection remain in place attached to the surface of the flask without the necessity for an agarose overlay, enabling foci to be counted easily.

This simple, quick and highly visual assay for infectious HIV-1 will be of immediate use as an alternative to reverse transcriptase or immunoassay for quantitation during laboratory maintenance of HIV infected cultures, and for detection and quantitiation of newly isolated HIV strains. It could also be useful for detection of varaemia in AIDS patients during progression of the disease, or following chemotherapy and, unlike immunoassay, would distinguish viable HIV from dead or defective virus particles.

Clone 22 (4) gave a linear response in terms of numbers of blue foci or units of β-galactosidase activity when titrated against serial dilutions of HIV-1.

Because of its simplicity the assay will be ideal for examination of the efficacy of antiviral agents directed against any stage of the HIV-1 life cycle. Many different compounds would be easily screened simultaneously for inhibition of virus growth using this method. Superficially similar assays based on the combined expression of LTRLacZ and the HIV transactivator tat in a HeLa cell line (Bachelor, 1989) or a dog cell line (Hasler et al., 1989) could only screen compounds directed against tat.

The formation of large clearly visible foci of infection should also permit use of these indicator cell lines for plaque purification (cloning) of HIV strains, a procedure that has previously been extremely difficult to perform. It should be noted however that recent data from Chesebro's laboratory (Chesebro, 1989) have shown that an amphotropic murine retrovirus was introduced into the $CD4^+$ HeLa cell line (HT4-6C) along with the $CD4^+$ antigen. HIV virions produced in HT4-6C could acquire murine retroviral envelope proteins, altering their structure and potential host range. Lusso et al. (1990) have demonstrated expanded HIV-1 cellular tropism by phenotypic mixing with endogenous murine retroviruses.

Although this phenomenon would not affect the utility of our HT4-6C based indicator cell line for identification and quanttiation of HIV infection, its use for plaque purification of virus would not be reliable. However, the 'Axel' HeLa-T4 cell line (Madden et al., 1986) has been shown not to be contaminated with the murine retrovirus (Chesebro 1989). Therefore, our indicator cell lines based on this $CD4^+$ HeLa cell line should be applicable for plaque purification experiments.

To construct indicator cell lines that are likely to be more sensitive to infection by HIV-1, we propose to introduce the LTRLacZ expression vectors that we have made into two new adherent $CD4^+$ cell lines, a $CD4^+$ rhabdomyosarcoma cell line (TE

671), (a gift of P. Clapham, Chester Beatty Laboratories), and an adherent human T lumphoid cell line (CEM-CL10), (Tremblay et al., 1989). The latter strain has been shown to be highly susceptible to infection by both laboratory and clinical strains of HIV-1. We also intend to construct non adherent lymphoid $CD4^+$ cell lines containing the LTRLacZ expression systems.

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CLAIMS

1. A plasmid comprising (1) a replicon for extrachromosomal expression in eukaryotic cells of a linked gene, (2) a gene which codes for a dominant selectable marker and (3) a transactivatable promoter element of an HIV Long Terminal Repeat (LTR) operably linked to the LacZ gene in a manner effective to provide transcription and expression of the LacZ gene following transactivation of the promoter element.

2. A plasmid according to claim 1, wherein the LTRLacZ construct (3) is in the same relative orientation as the replicon (1).

3. A plasmid according to claim 1 or 2, wherein the marker gene (2) is in the same relative orientation as the replicon (1).

4. A plasmid according to claim 1, 2 or 3, wherein the replicon (1) comprises an origin of eukaryotic cell replication and a nuclear antigen gene.

5. A plasmid according to claim 4, wherein the replicon (1) is from human papovavirus BK or Epstein Barr Virus.

6. A plasmid according to any preceding claim, wherein the marker gene (2) is a hygromycin or neomycin resistance gene.

7. A plasmid according to any preceding claim, wherein the LTRLacZ construct (3) includes a polyadenylated (poly A) termination sequence.

8. A plasmid according to claim 7, wherein the poly A sequence is from cytomegalovirus immediate-early gene.

9. A plasmid according to any preceding claim, which is bacterially replicable.

10. A plasmid according to claim 1 substantially as shown in Figure 11.

11. A plasmid according to claim 1 substantially as shown in Figure 9, but with the EBNA nuclear antigen in the opposite orientation to that shown.

12. HIV-responsive cells transfected with a plasmid claimed in any preceding claim.

13. Cells according to claim 12 which are CD4-positive.

14. Cells according to claim 13 which are HeLa cells.

15. A method of assay of human immunodeficiency virus which comprises incubating a sample, suspected of being HIV-infected, to cells according to claim 12, 13 or 14 under conditions allowing transactivation of the promoter element of the LTRLacZ construct by HIV present in the sample, and assaying the beta-galactosidase produced.

16. A method according to claim 15, wherein the beta-galactosidase assay is colorimetric.

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